519 Rec'd PCT/PTO 31 JUL 2001

FORM PTO-1390
(REV 11-98)

ATTORNEY DOCKET NUMBER
0558-4011

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)
TBA 09/890478

INTERNATIONAL APPLICATION	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP00/00455	21 January 2000	4 February 1999

TITLE OF INVENTION
PH-SENSITIVE AMPEROMETRIC BIOSENSOR

APPLICANT(S) FOR DO/EO/US
Andrea Pizzariello, Miroslav Stredansky, Silvia Stredanska and Stanislav Miertus

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. ☐ This is **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C 371.

3. ☒ This express request to begin national examination procedures (35 U S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S. C. 371 (b) and PCT Articles 22 and 39 (1).

4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))

   a. ☐ is transmitted herewith.
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).

6. ☐ A translation of the International application into English (35 U.S.C. 371(c)(2)).

7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U S.C. 371(c)(3))

   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.

8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). (executed)

10. ☒ A copy of the annexes to the International Preliminary Examination Report under PCT Article 36 is enclosed (35 U S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included.

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. ☒ A FIRST preliminary amendment.

    ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☐ A substitute specification.

15. ☐ A change of power of attorney and/or address letter.

16. ☒ Other items or Information:

- Copy of Notice Informing the Applicant of the Communication of the International Application to the Designated Offices.
- Copy of PCT Request
- Copy of International Application Published Under the Patent Cooperation Treaty (PCT) No. WO 00/46393
- Information Concerning Elected Offices Notified of Their Election
- Check in the amount of $470.00
- Return postcard.

JC18 Rec'd PCT/PTO 3 1 JUL 2001

U.S. APPLICATION NO (if known, see 37 C F R 1 51)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NO
TBA 09/890478	PCT/EP00/00455	0558-4011

17. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):	CALCULATIONS	PTO USE ONLY
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO and International Search Report not prepared by the EPO or JPO $1000.00		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $860.00		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2) paid to USPTO $710.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33 (1) - (4) $690.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1) - (4) $100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 860.00	
Surcharge of **$130** for furnishing the oath or declaration later than ☐20 ☐30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$-- 0.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	16-20	0	X $18.00	$ 0.00
Independent claims	1-3	0	X $80.00	$-- 0.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $270.00	$ 0.00
TOTAL OF ABOVE CALCULATIONS =				$ 860.00
Reduction of ½ for filing by small entity, if applicable. A Small Entity Statement must also by filed (Note 37 CFR 1.9, 1.27, 1.28). Applicant asserts it is a SMALL ENTITY.				$-430.00
SUBTOTAL =				$ 860.00
Processing fee of **$130.00** for furnishing the English translation later than ☐20 ☐30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$-- 0.00
TOTAL NATIONAL FEE =				$ 430.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$ 40.00
TOTAL FEES ENCLOSED				$ 470.00
			Amount to be refunded:	$
			charged	$

a. ☒ A check in the amount of $900.00 cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 13-4500 in the amount of to cover the above fees.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 13-4500, ORDER NO. 0558-4011 . A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
Morgan & Finnegan LLP
345 Park Avenue
New York, NY 10154-0053
Telephone: 212-758-4800
Telecopier: 212-751-6849

William S. Feiler

Registration Number 26,728

U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NO.
TBA 09/890478	PCT/EP00/00455	0558-4011

Rec'd PCT/PTO 31 JUL 2001

17. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):	CALCULATIONS	PTO USE ONLY
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO and International Search Report not prepared by the EPO or JPO..... $1000.00		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO...$860.00		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2) paid to USPTO.....$710.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33 (1) - (4).....$690.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1) - (4).....$100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 860.00	
Surcharge of **$130** for furnishing the oath or declaration later than ☐20 ☐30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$-- 0.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	16-20	0	X $18.00	$ 0.00	
Independent claims	1-3	0	X $80.00	$-- 0.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $270.00	$ 0.00	
TOTAL OF ABOVE CALCULATIONS =				$ 860.00	
Reduction of ½ for filing by small entity, if applicable. A Small Entity Statement must also by filed (Note 37 CFR 1.9, 1.27, 1.28). Applicant asserts it is a SMALL ENTITY.				$-430.00	
SUBTOTAL =				$ 860.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐20 ☐30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$-- 0.00	
TOTAL NATIONAL FEE =				$ 430.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property =				$ 40.00	
TOTAL FEES ENCLOSED				$ 470.00	
			Amount to be refunded:	$	
			charged	$	

a. ☒ A check in the amount of $900.00 cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 13-4500 in the amount of to cover the above fees.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 13-4500, ORDER NO. 0558-4011 . A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
Morgan & Finnegan LLP
345 Park Avenue
New York, NY 10154-0053
Telephone: 212-758-4800
Telecopier: 212-751-6849

William S. Feiler

Registration Number 26,728

640209 v1

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s)	:	Pizzariello et al.	Group Art Unit	: TBA
Int'l Application	:	PCT/EP00/00455	Examiner	: TBA
Filed	:	21 January 2000		
For	:	**PH-SENSITIVE AMPEROMETRIC BIOSENSOR**		

PRELIMINARY AMENDMENT

Commissioner Of Patents
Washington, D.C. 20231

Sir:

Please enter the following Preliminary Amendment prior to consideration of the application on the merits. A copy of the amended claims showing insertions and deletions is included in an Appendix to this paper.

IN THE CLAIMS

Please amend claims 6, 7, 9, 10, 11 and 16 to read as follows:

6. The biosensor system according to claim 1, wherein said pH-sensitive redox compound (b) is in the form of a monomer, oligomer or polymer.

7. The biosensor system according to claim 6, wherein said pH-sensitive redox compound (b) is selected among the pH indicators, phenoxazines and phenothiazines dyes, and natural antioxidants.

9. The biosensor system according to claim 1, wherein said working electrode (c) is a solid composite electrode, or platinum electrode, or gold electrode, or mercury electrode or glassy carbon electrode.

10. The biosensor system according to claim 1, wherein said reference electrode (d) is selected from the group consisting of Ag/AgCl and calomel electrodes.

11. A method for the determination of analytes characterized by the use of a biosensor as claimed in claim 1.

16. A method according to claim 11, wherein said determination of analytes is performed in the fields of human and veterinary diagnostics, industrial processes, agro-food industry, pharmaceutical industry, or environmental monitoring.

REMARKS

Applicants have amended the claims to eliminate multiple dependent claims to minimize cost associated with filing this national phase application and to conform the claims to the standard of practice before the U.S. Patent and Trademark Office. Specific additions and deletions to the claims are shown in an Appendix to this paper. The amendment to the claims does not introduce new matter. Entry of this Preliminary Amendment is respectfully requested.

Respectfully submitted,

MORGAN & FINNEGAN, L.L.P.

Dated: July 31, 2001

William S. Feiler
Reg. No. 26,728

MORGAN & FINNEGAN, L.L.P.
345 Park Avenue
New York, New York 10154
(212) 758-4800
(212) 751-6849 Facsimile

APPENDIX

6. The biosensor system according to [claim 1-5] claim 1, wherein said pH-sensitive redox compound (b) is in the form of a monomer, oligomer or polymer.

7. The biosensor system according to [claims 1,-6] claim 6, wherein said pH-sensitive redox compound (b) is selected among the pH indicators, phenoxazines and phenothiazines dyes, and natural antioxidants.

9. The biosensor system according to [claims 1-8] claim 1, wherein said working electrode (c) is a solid composite electrode, or platinum electrode, or gold electrode, or mercury electrode or glassy carbon electrode.

10. The biosensor system according to [claims 1-9] claim 1, wherein said reference electrode (d) is selected [in] from the group consisting of Ag/AgCl and calomel electrodes.

11. A method for the determination of analytes characterized by the use of a biosensor as claimed in [claims 1-10] claim 1.

16. [Use of the biosensor system as claimed in claims 1-10 for the amperometric detection of analytes in] A method according to claim 11, wherein said determination of analytes is performed in the fields of human and veterinary diagnostics, industrial processes, agro-food industry, pharmaceutical industry, or environmental monitoring.

101 Rec'd PCT/PTO 31 JUL 2001
09/890478

PH-SENSITIVE AMPEROMETRIC BIOSENSOR

Field of the invention

The present invention relates to the field of electrochemical analysis. It refers specifically to systems for the electrochemical detection of analytes based on the activity of biocatalysts. The object of this invention is a new group of biosensors and their use in a method for the detection of analytes.

Prior art

A biosensor is a device that embodies a biological sensing element that is either connected to or inserted into a transducer. The aim is the production of electronic signals proportional to the concentration of the specific substance that has to be determined.

The advent of biosensors has provided an interesting alternative to conventional laboratory analysis. Due to their simple manipulation, compactness and versatility of use, biosensors allow for easy performances of on-site tests. Specific and sensitive devices have been used in medical diagnostics, quality assessment of food, environmental monitoring, fermentation techniques, analytical control and so on.

Electrochemical biosensors, specifically the amperometric ones, play a significant role in the use of these detection devices.

Amperometric biosensors produce a linear signal and are featured by high sensitivity. Under favourable conditions, analyte concentrations ranging from $1x10^{-8}$ to $1x10^{-9}$ M can be detected and a dynamic range from three to four order of magnitudes can be easily obtained (G.S. Wilson, in "Biosensors, Fundamentals and Applications, A.P.F. Turner, I. Karube and G.S. Wilson Ed., Oxford Univ. Press, 165-179, 1987).

The first generation of amperometric biosensors is based on the oxidation of the analyte by oxidases (biocatalysts) using oxygen as an electron acceptor. As a consequence, either the reduction in the oxygen concentration or the increase in the produced hydrogen peroxide concentration are measured by an electrode in the form of current that is proportional to the analyte concentration.

In the second generation systems, the enzyme performs the first redox reaction with the substrate (the analyte) but is then reoxidised by a redox mediator as

opposed to oxygen; the mediator is then oxidised by the electrode and the corresponding amperometric signal is measured. Many examples of mediators containing biosensors are quoted in a review by Gorton (Electroanalysis, 7, 23-45, 1995).

Since the redox mediators shuttle electrons that come to the redox centre of the biocatalyst from the substrate to the working electrode, a limitation inherent in these amperometric biosensors consists in the use of the biocatalysts belonging to the oxidoreductase group. As a consequence, these biosensors can detect of a limited group of analytes.

A certain number of enzymes belonging to the groups of hydrolases, transferases, oxidoreductases, lyases, ligases, and in particular decarboxylases, phosphorylases, esterases, phosphatases, deaminases, kinases, changes the concentration of H^+ ions (by either consumption or production) by their biocatalytic interaction with a substrate and this change depends on the substrate concentration. These biocatalysts, combined with a suitable potentiometric transducer (for example the typical glass pH electrode or with the solid and liquid membrane pH electrode) are used for the implementation of potentiometric biosensors. Examples of analytes that are determined by these biosensors are urea, penicillin, glucose, malate (S.S. Kuan and G.G. Guibault, In: Biosensors, Fundamentals and Applications, A.P.F. Turner, I. Karube and G.S. Wilson Ed., Oxford Univ. Press, 135-152, 1987; Palleschi et al., Talanta, 41, 917-923, 1994). The disadvantages of these biosensors consist in a logarithmic response and in a low sensitivity. Their useful analytical range is generally from $1x10^{-1}$ to $1x10^{-4}$ M, exceptionally to $1x10^{-5}$ M.

Another group of potentiometric biosensors uses a combination of biocatalysts that modify their pH when interfaced with ion-sensitive field effect transistors (ISFET). ISFET are prepared with a manufacturing procedure based on silicon where the silicon nitride layer deposited on the surface is mostly used as a pH-sensitive transducer. Some examples consist in biosensors for the detection of urea, ATP, penicillin, glucose and acetylcholine (G.F. Blackburn, In: Biosensors, Fundamentals and Applications, A.P.F. Turner, I. Karube and G.S. Wilson Ed., Oxford University Press, 481-530, 1987).

The drawbacks inherent in these biosensor consist in a low sensitivity (measurable response in concentration range from $1x10^{-1}$ to $1x10^{-4}$ M), high costs and a complex manufacturing procedure.

Recently, a new group of electrochemical biosensors based on the combination of a biocatalyst that modifies the pH and a conductometric transducer (A.Q. Contractor et al., Electrochim. Acta, 39, 1321-1324, 1994; J.M. Goncalves Laranjeira et al, Anal.Lett. 30, 2189-2209,1994; Nishizawa et al., Anal. Chem., 64, 2642-2644, 1992) has been described. This new kind of biosensors exploits the pH effect on the electric properties of a conductive polymer (polyaniline, polypyrrole) deposited on the electrode surface. They consist in two platinum electrodes that are placed at a distance of several μm and covered by the conductive polymer film and an enzymatic membrane. With this kind of biosensor it is possible to detect analytes such as urea, glucose, lipids, haemoglobin and penicillin. These biosensors provide a fast response and an improved sensitivity with respect to the potentiometric biosensors (the useful analytical range goes from $1x10^{-1}$ to $1x10^{-5}$ M, in the best cases $1x10^{-6}$M); however, their sensitivity is still far from the one that can be obtained with amperometric biosensors. Moreover, they require an accurate and expensive manufacturing procedure.

As a consequence, in view of the drawbacks listed previously, it is necessary to identify alternative electrochemical biosensors with higher sensitivity and an easier manufacturing procedure.

Summary of the invention

The present invention describes a new electrochemical biosensor comprising (i) a biocatalyst producing a pH change when interacting with the analyte to be determined and (ii) a compound exhibiting different redox properties both in its protonated and non-protonated forms (pH-sensitive redox compound).

The elements described above are integrated in a biosensor system composed of a working electrode and a reference electrode connected to an ammeter. When the analyte is present, the system produces a current change that is proportional to the concentration of the analyte. The biosensors described herein can be used in the accurate detection of a wide range of analytes. They can be used in diagnostics, industrial processes, food and feed quality control, biotechnology,

pharmaceutical industry, environmental monitoring and so on.

Description of figures

Figures 1-7 show the current change dependency on pH at suitable constant potentials using several pH-sensitive redox compounds and various electrodes as described in Examples 1-7.

Figure 1: platinum electrode; dissolved hematein at the concentration of 0.5 mM (curve a) and 2.5 mM (curve b);

Figure 2: dissolved hematein; carbon paste electrode (curve a) and solid composite electrode (curve b);

Figure 3: golden electrode with methylene blue monolayer;

Figure 4: solid composite electrode; dissolved hematoxylin (curve a), dissolved quercitin (curve b), dissolved harmaline (curve c);

Figure 5: solid composite electrode with electropolymerised orto-phenylendiamine;

Figure 6: platinum electrode with electropolymerised pyrogallol;

Figure 7: solid composite electrode modified with laurylgallate;

Figures 8-16 show the calibration curves of several analytes measured with the biosensors of the invention as described in the Examples 8-17.

Figure 8: biosensor for the detection of urea, dissolved hematein, platinum electrode (curve a) or solid composite electrode (curve b);

Figure 9: biosensor for the detection of urea, dissolved hematein, solid composite electrode containing urease, in the presence of either 5 mM (curve a) or 1 mM (curve b) phosphate buffer;

Figure 10: biosensor for the detection of urea, solid composite electrode modified with alkylgallate;

Figure 11: biosensor for the detection of oxaloacetate, dissolved hematein, solid composite electrode;

Figure 12: biosensor for the detection of glucose, solid composite electrode modified with poly(ortho-phenylendiamine) film;

Figure 13: biosensor for the detection of hydrogencarbonate, dissolved hematein, platinum electrode;

Figure 14: biosensor for the detection of penicillin, dissolved hematein, platinum electrode;

Figure 15: biosensor for the detection of ATP, dissolved hematein, platinum electrode;

Figure 16: biosensor for the detection of urea, golden electrode with methylene blue monolayer.

Detailed description of the invention

The object of the present invention is an amperometric biosensor system for the detection of analytes comprising:

at least one biocatalyst producing a pH change by its interaction with the analyte to be determined;

at least one compound exhibiting different redox properties both in its protonated and non-protonated forms. Said compound will be hereinafter indicated as "pH-sensitive redox compound";

a working electrode;

a reference electrode.

The electrodes at c) and d) are connected through an ammeter.

In one embodiment of this invention, the biocatalyst (a) and the pH-sensitive redox compounds (b) are contained in the working electrode; as an alternative, one or more of these components are present in the measuring solution in which the electrodes are immersed.

The biosensor of this invention can optionally be covered with a suitable semipermeable membrane.

The working principle of these biosensors is described hereinafter. The electrodes are immersed into a measuring solution and a suitable potential is applied between them. The electrode reaction is carried out up to reaching the equilibrium between the oxidised and the reduced form of the pH-sensitive redox compound (b). This electrochemical reaction is accompanied by an electron flow measured in the form of electric current by the ammeter. Up to this stage, the biocatalyst (a) is not involved. Once the sample containing the analyte is added to the solution, the biocatalyst/analyte reaction takes place and the pH is modified accordingly; the pH variation modifies the equilibrium of the protonated/non-protonated forms of the redox compound (b). Since these forms of the redox compound exhibit different redox properties, any changes in their concentration produce a current change at

the constant potential applied. The current change is monitored by the ammeter and depends on the substrate concentration.

As regards the nature of this biocatalyst (a), it can be any biological entity capable of interacting with the analyte to be determined and causing a pH variation as a result of such interaction. As a matter of fact, any biocatalyst reacting with its normal substrate either producing or consuming H^+ ions can be used as a biocatalyst for the detection of that substrate. Suitable biocatalysts are, for example, enzymes catalyzing reactions that involve either the production or the consumption of H^+ ions; typical examples are hydrolases, oxidoreductases, transferases, lyases, ligases and preferably phosphorylases, decarboxylases, esterases, proteinases, deaminases, amidases, phosphatases, and synthetases. Other examples of biocatalysts with the same features are to be found among imunoproteins, nucleic acids, sinzymes, catalytic antibodies.

Other pH changing biocatalysts can be found among biological structures or biological aggregates such as cells or cell fragments, tissues, organelles and their fragments, fractions, homogenates, extracts, lysates.

It is possible to use one single biocatalyst or a mixture of two or more of them.

The choice of the suitable biocatalysts is determined by the nature of the analyte itself, according to the principle whereby any analyte works as a substrate for a given biocatalyst: for example, esterases are indicated for the analytical detection of esters; decarboxylases are used for the detection of carboxylic acids, deaminases for amines and so on.

Examples of preferred biocatalysts for the present invention are: urease, oxalacetate decarboxylase, glucose oxidase, carbonic anhydrase, penicillinase, apyrase for the detection respectively of urea, oxalacetate, glucose, hydrogencarbonate, penicillin, ATP.

In the biosensors of the invention, the biocatalysts (a) can be incorporated in the working electrode or otherwise can be present in the measuring solution in either a dispersed or soluble form.

The incorporation of said biocatalyst in the working electrode is particularly suited for the preparation of composite biosensors: these biosensors are especially preferred.

Said biocatalysts can also be applied onto the surface of the working electrodes. In this case, they are normally immobilized by means of physical or chemical methods. The preferred methods for immobilization consist in one or more among: covering with a semipermeable membrane, entrapping in a polymer or in a gel layer, crosslinking with bifunctional agents, covalent binding, adsorption, and immobilization in the outer membrane.

The biocatalyst is normally placed in the measuring solution by dissolving the biocatalyst in the solution or by dispersing it homogeneously. This is particularly indicated for disposable thick-layer biosensors, where the biocatalyst is dissolved in the whole volume of the sample added. It is devised specifically for biosensors that determine polymeric analytes since it avoids steric hindrances that could occur when the biocatalyst is immobilized. Another possible way of placing the biocatalyst in the biosensor of the invention consists in its immobilization in a small bioreactor inserted in front of the working electrode when the flow system is applied.

When the activity of the biocatalyst requires the presence of a cofactor, for example a coenzyme or an activator, the biosensors of this invention include also said cofactor. The cofactor is preferably placed together with the biocatalyst, i.e. they are either placed onto the electrode surface, or in the electrode body or in the solution.

A further element of the biosensor system according to the present invention is represented by the pH-sensitive redox compound (b). These are compounds that are present in solution in equilibrium beween the protonated and the non-protonated form having different redox potentials.

The pH-sensitive redox compounds are selected in the group consisting of cyclic hydrocarbons containing from 4 to 30 carbon atoms and susbstituted with at least one group selected from -OH, -SH, - NH_2, =O, =S, =NH, -OR_1, -SR_1, -NHR_1, -NR_1R_2, =NR_1, where R_1 and R_2 are hydrocarbon chains optionally further substituted, or selected in the group consisting of heterocyclic compounds containing from 3 to 30 carbon atoms and one or more heteroatoms selected in the group consisting of N, S, O, Se, Te, B, P, As, Sb, Si, optionally substituted with a group selected from -OH, -SH, - NH_2, =O, =S, =NH, -OR, -SR_1, -NHR_1, -

NR_1R_2, $=NR_1$, where R_1 and R_2 are independent hydrocarbon chains. These compound can be selected in the form of either monomer, or oligomer or polymer. The above mentioned compounds can be used either alone or in a mixture with one or more of them.

The preferred classes of pH-sensitive redox compounds are indicators of pH (ie. hematoxylin, hematein), phenoxazine and phenothiazine dyes (i.e. methylene blue), natural antioxidants (i.e. quercitin, flavonoids, alkylgallates) polymerised ortho-phenylendiamine or para-phenylendiamine.

According to the invention, the pH-sensitive redox compound is present in the working electrode or dissolved in the measuring solution. The pH sensitive redox compounds that are water soluble are preferably added to the solution; those insoluble in water are preferably used to modify the working electrode.

When present in the working electrode, the pH-sensitive redox compound can be deposited onto its surface in a free form; otherwise, it can be chemically or physically bound (immobilized) onto the working electrode surface; or alternatively it can be a component of the body of a composite working electrode .

If the pH-sensitive redox compound is either a polymer or an oligomer, this can be prepared also *in situ* on the working electrode by chemical or physical polymerization, preferably by radical polymerization, electropolymerization or photopolymerization.

Among the redox compounds quoted above, phenothiazines dyes and poly(ortho-phenylendiamine) are particularly suited to be either physically or chemically bound to the electrode surface. Hematein, hematoxillin, phenothiazines dyes and quercitin are particularly indicated to be added to the measuring solution. Alkylgallates are preferably suited to be incorporated in the biosensor's body as components of a composite working electrode.

Several working electrodes can be used as element (c) of the biosensor system of this invention. Said working electrodes are selected in the group consisting of the typical working electrodes used in amperometry (like, for example, platinum, gold, mercury, glassy carbon electrodes) or by composite electrodes (such as for example the solid composite electrodes).

For the purpose of the present invention, by the term "solid composite electrodes"

are meant the electrodes described in WO 97/02359, hereby incorporated by reference.

Similarly, reference electrodes useful as element (d) of the biosensor of this invention are commonly available in amperometry. The preferred reference electrodes are standard calomel electrodes (SCE) and Ag/AgCl electrodes. The Ag/AgCl electrodes are particularly suitable because they can be designed in various forms like for example wire disc, layer or bar.

The working potential to be applied between the two electrodes is preferably about 0.0 mV or it is negative (versus Ag/AgCl reference electrode). The application of this potential significantly reduces possible electrochemical interferences deriving from easily oxidizable interfering compounds present in real samples.

Differently from the typical amperometric biosensors, where the measurements are carried out in strongly buffered solutions requiring a constant pH, in the present invention the measurements are carried out in non-buffered solutions or in solutions having a low buffering capacity. If a solution having low buffering capacity is used, the preferred concentration of the buffering compounds ranges from 0.5 to 20 mM.

The term "measuring solution" used in this invention is not strictly limited to systems where all components are dissolved; it also includes liquid systems where at least part of the components are contained in a homogeneously dispersed status such as suspensions, emulsions and so on. The biosensor implemented as described in the present invention can amperometrically determine many more analytes than was possible so far.

The biosensor system according to the present invention shows better performances in term of detection limit, linearity of the output signal, rapidity of response, selectivity and stability of those reported in literature. Besides the good specificity and sensitivity, a simple manufacturing procedure and a versatile design represent also relevant advantages of the biosensor system of the present invention. The biosensor's sensitivity described hereinafter (see Examples) ranges from 0.1 to 5 $\mu A\ mM^{-1}\ cm^{-2}$ and the detection limits range from $1x10^{-5}$ to $1x10^{-7}$ M.

The biosensors of the present invention are versatile with respect to the biocatalyst, the pH-sensitive redox compounds, the working and reference electrodes and the setting of the biosensor. They can also have a good variability in the design and can be shaped in many different forms such as for example, strips, tips and needles. Disc, tube, wire, thick layer, thin layer and other forms of the electrodes fit perfectly in the biosensor described in this invention. The preparation of microelectrodes according to the present invention is also possible. The biosensor system according to the present invention can be profitably used in human and veterinary diagnostics, industrial processes, agro-food industry, biotechnology, pharmaceutical industry, environmental monitoring and so on. All these possible uses are included in the present invention.

A further embodiment of the present invention concerns a method for the determination of the analytes concentration characterised by the use of the new biosensors described previously.

A preferred method for the determination includes the following steps:

(a) placing the electrodes in a measuring solution;

(b) applying a suitable potential between the electrodes;

(c) measuring a background current;

(d) adding to the solution the sample containing the analyte to be determined;

(e) measuring the current change that is proportional to the analyte concentration;

(f) optionally subtracting the current change measured with a blank electrode from the value obtained in (e).

Step (f) is added so as to eliminate possible interferences. The blank electrode differs from a normal working electrode as described so far, only in as much as it either contains said biocatalysts in a non-active form or it does not contain them at all. The procedure for obtaining a current change measured with the blank electrode is the same as the one described in steps (a)- (e).

All readings are carried out when the sample is uniformly diluted in the measuring solution and the signal is stable.

As described above, the invention is compatible with several biosensor designs, such as tips, needles, strips and so on. Some of these forms (see strip biosensor) work in absence of a measuring solution and react immediately upon contact with

the sample containing the analyte. This contact occurs for example when a drop of the sample containing the analyte is added to the biosensor, on the biosensor or by plunging the biosensor itself in the solution. In these cases, the method for the detection of the analyte is modified in the following way:

(a) applying a suitable potential between the electrodes;

(b) measuring a background current;

(c) contacting the biosensor with the sample containing the analyte;

(d) measuring a current change that is proportional to the analyte concentration;

(e) optionally subtracting the current change measured with a blank electrode from the value obtained in (d).

The methods described above can be either qualitative (they determine the presence of the analyte in the solution) or quantitative (they determine the analyte concentration) since the current change is proportional to the analyte concentration.

So far, the biocatalyst has been defined to react positively with the analyte and thereby cause a pH change. In a further embodiment of this invention, the system identifies the presence of an analyte that is an inhibitor of the biocatalyst, thereafter called inhibiting-analyte. In this case, the interaction turns out to be negative and the current change depending on the extent of the inhibition will be proportional to the inhibiting-analyte concentration.

This aspect further broadens the range of analytes that can be identified with the biosensors of the present invention; each substance acting as the inhibitor of a pH-changing biocatalyst can be identified in this way.

With the purpose of implementing this aspect of the invention, the measurement method is partly modified by adding the normal substrate of the biocatalyst to the system before introducing the sample containing the inhibiting-analyte that has to be tested. As a consequence, the method comprises the following steps:

(a) placing the electrodes in a measuring solution;

(b) applying a suitable potential between the electrodes;

(c) adding the substrate of said biocatalyst to the measuring solution;

(d) measuring a background current;

(e) adding to the solution the sample containing the inhibiting-analyte to be

determined;

(f) measuring a current change that is proportional to the inhibiting-analyte concentration;

(g) optionally subtracting the current change measured with a blank electrode from the value obtained in (f).

If the biosensor's design (e.g. strip biosensor) allows to work in absence of a measuring solution, then the above method is modified as follows:

(a) applying a suitable potential between the electrodes;

(b) adding the substrate of said biocatalyst;

(c) measuring a background current;

(d) contacting the biosensor with the sample containing the inhibiting-analyte;

(e) measuring a current change that is proportional to the inhibiting-analyte concentration;

(f) optionally subtracting from the value obtained in (d) the current change measured with a blank electrode.

Step (c) is carried out either by adding a drop of the sample containing the inhibiting-analyte to the biosensor or by immersing the sample in the solution.

This method can be further used to determine the enzymatic activities. In such case, the current changes must be measured as time-dependent.

The present invention will now be illustrated with the following experimental examples, having no limitative function.

EXPERIMENTAL PART

EXAMPLE 1

Current change variation with pH in the presence of dissolved hematein by using a platinum electrode

Hematein (Fluka, Cat. No. 51230) is dissolved in 0.05 M phosphate buffer containing 0.1 M sodium chloride. The working platinum electrode and the SCE reference electrode are immersed in the solution and the current is measured by an Amel 559 amperometric detector (Amel Instruments, Milano, Italy) at the constant potential of 0.0 mV. The pH value decreases as 2M sulphuric acid aliquots are added and the corresponding current change is monitored. Meanwhile, the pH is measured by pH-meter (PHM 85, Radiometer, Copenhagen,

Denmark). The relationship between the current change and the pH for two concentrations of hematein (0.5 mM - curve a; and 2.5 mM - curve b) is illustrated in Figure 1.

EXAMPLE 2

Current change variation with pH in the presence of dissolved hematein by using composite electrodes

The carbon paste electrode is prepared by mixing, under vigorous stirring, 7 parts (w/w) of graphite (Fluka, Cat. No. 50870) with 3 parts (w/w) of paraffin oil (Fluka, Cat. No. 76235) in a mortar. The mixture is introduced into a plastic tube (inner diameter: 2mm) equipped with a brass rod. The solid composite electrode is prepared by mixing vigorously 2 parts (w/w) of graphite with 3 parts (w/w) of melted n-eicosane (Sigma, Cat. No. E-9752) at 45°C. This mixture is introduced into a plastic tube (inner diameter: 2mm) equipped with a brass rod. Both electrodes are smoothed with a sheet of paper before use. The electrochemical measurements are carried out as described in Example 1 with 0.5 mM hematein and the current changes obtained are reported in Figure 2 (curve a – carbon paste electrode, curve b - solid composite electrode)

EXAMPLE 3

Current change variation with pH by using a golden electrode modified with methylene blue.

The newly polished golden electrode (Amel Instruments) is immersed in a 0.5 mM methylene blue solution (Aldrich, Cat. No. 86, 124-3) for 12 hours. Then, the electrode is accurately rinsed with deionized water. The electrochemical measurements are carried out as described in Example n.1, by using a working potential of -100 mV (versus SCE). The results are reported in Figure 3.

EXAMPLE 4

Current changes variation with pH by using a solid composite electrode in the presence of dissolved hematoxylin, quercitin and harmaline.

The solid composite electrodes are prepared as described in Example 2. The pH is measured in 0.5 mM solutions of hematoxylin, quercitin, harmaline by using the buffer described in Example n. 1. The working potential for hematoxylin and quercitin is 0.0 mV (versus SCE), while for harmaline is 600 mV. The results are

illustrated in Figure 4 (curve a - hematoxylin; curve b - quercitin; curve c - harmaline).

EXAMPLE 5

Current change variation with pH by using a solid composite electrode the surface of which has been modified with a poly(ortho-phenylendiamine) film.

The solid composite electrode is prepared by mixing vigorously graphite with melted n-eicosane (weight ratio1:1) at 45°C. The mixture obtained in this way is introduced into a plastic tube (inner diameter 2mm) equipped with a brass rod. A poly-(ortho-phenylendiamine) film is deposited onto the polished electrode surface by means of electrochemical polymerization of ortho-phenylendiamine monomer (Sigma, Cat. No. P-9029) in aqueous solution. This process is carried out in the following way: the scanning of the electrode potential is repeated 30 times from -0.5 mV to 0.7 mV (versus SCE) at 50 mVs^{-1} in oxygen-free 0.1 mM acetate buffer at pH 5.0 which contains 0.5 mM ortho-phenylendiamine under inert atmosphere. The modified electrode is then thoroughly rinsed with the phosphate buffer. This biosensor is then tested at different pH values of a solution and the current change is measured according to the procedure described in Example 1. The working potential is -600 mV. The results are illustrated in Figure 5.

EXAMPLE 6

Current change variation for a platinum electrode the surface of which is modified with polypyrogallol.

The polypyrogallol film is deposited upon the newly polished surface of the platinum electrode by electrochemical polymerization of 25 mM of pyrogallol (Aldrich, Cat. Mo. 25.400-2) in aqueous solution containing 0.15 M phosphate buffer (pH 7.0) and tetraethylamonium tetrafluoroborate 0.1 M (Aldrich, Cat. No. 24, 214-4). The scanning of the potential electrode is repeated three times from 0.0 V and 1.1 V (versus SCE) at 50 mVs^{-1}. The modified electrode is then rinsed thoroughly with the phosphate buffer. This biosensor is tested at the different pH values of a solution and the current change is measured with the same procedure as described in Example n. 1. The working potential is 200 mV. The results are shown in Figure 6.

EXAMPLE 7

Variation of the current changes for a solid composite electrode modified with lauryl gallate.

The graphite powder is modified as follows: 100 mg of lauryl gallate (Fluka, Cat. No. 48660) are dissolved in 2 ml of acetone and 400 mg of modified graphite are added to the solution. The mixture is stirred up to being made homogeneous and acetone is then evaporated under forced air flow at room temperature. 100 mg of lauric acid (Fluka, Cat. Mo. 61610) and 150 mg of 2-hexadecanone (Fluka, Cat. No. 69250) are dissolved in a porcelain dish at 50 °C and stirred vigorously with 250 mg of the modified graphite. A plastic tube (inner diameter 2 mm) equipped with a brass rod is filled with this mixture; the electrode material then solidifies at room temperature. The electrode surface is smoothed with sand paper and cleansed with a sheet of common paper. The current change dependence on the pH of the electrode modified by lauryl gallate is measured with the same procedure as the one described in Example n.1. The working potential is 200 mV. The results are illustrated in Figure 7.

EXAMPLE 8

Preparation of the biosensor for the determination of urea based on a platinum electrode modified with urease and dissolved hematein

A solution (2 µl, 10 mg/ml) of urease (EC 3.5.1.5., Sigma, Cat. No. U-0376) is applied onto the surface of the platinum electrode. After drying at room temperature, the electrode is covered with a dialysis membrane (Spectra/Por MWCO 6,000 - 8,000), fixed by means of an O-ring. The biosensor is immersed in 1 mM phosphate buffer (pH=7.35) containing 0.5 mM hematein and 0.1 mM sodium chloride. Hence, the biosensor is polarized at 0.0 mV (versus SCE) and a few aliquots of urea solution (5 mg/ml) are added to the measuring buffer. The relationship between the urea concentration and the current change is reported in Figure 8 (curve a).

EXAMPLE 9

Preparation of the biosensor for the determination of urea based on the solid composite electrode modified with urease and dissolved hematein.

The solid composite electrode is prepared as described in Example 2. Urease (2 µl, 10 mg) is applied onto the clean electrode surface. After drying, the electrode is

covered with a dialysis membrane (Spectra/por MWCO 6,000 - 8,000) by means of an O-ring. The biosensor is then immersed in 1mM phosphate buffer (pH = 7.35) containing 0.5 mM hematein and 0.1 M sodium chloride. The electrode is then polarized at 0.0 mV (versus SCE). A few aliquots of urea standard solutions (5mg/ml) are added to the measuring buffer. The relationship between the urea concentration and the current change is shown in Figure 8 (curve b).

EXAMPLE 10

Preparation of the biosensor for detection of urea based on the bulk modified solid composite electrode and dissolved hematein

The graphite powder is modifed in the following way: 97 mg of graphite powder are added to 0.5 ml urease aqueous solution (6 mg/ml). The mixture is accurately mixed to obtaining a homogeneous mixture and water is then gently evaporated. 50 mg of the modified graphite are mixed with 50 mg of 2-hexadecanone at 50 °C and the mixture obtained is poured into a plastic tube (inner diameter 2 mm) equipped with a brass rod; the mixture is then cooled down at room temperature. The electrode is smoothed with a sheet of paper and covered with a dialysis membrane (Spectra/Por MWCO 6,000 - 8,000). The biosensor is immersed in the phosphate buffer (1 or 5mM, pH 7.50) containing 0.5 mM hematein and 0.1 M sodium chloride. It is then polarized at 0.0 mV (versus SCE). A few aliquots of urea standard solution (5 mg/ml) are added to the measuring buffer. The current changes are recorded and the results are illustrated in Figure 9, where curve b) refers to 1 mM phosphate buffer.

EXAMPLE 11

Preparation of the biosensor for the determination of urea by using a solid composite electrode modified with urease and containing lauryl gallate.

The graphite powder is modified in the following way: 20 mg of lauryl gallate are dissolved in 0.5 ml of acetone and 90 mg of graphite are added to the solution. The mixture then is stirred up to making it homogeneous and acetone is evaporated under forced air at room temperature. 40 mg of 2-hexadecanone and 5 mg of stearic acid (Aldrich, Cat. No. 26, 838 - 0) are dissolved in a porcelain dish at 55 °C and mixed vigorously with 55 mg of the modified graphite quoted above. The mixture is then poured into a plastic tube (inner diameter: 2mm) equipped with

a brass rod. Urease (1μl, 30 mg/ml) is applied onto the newly cleansed electrode surface. After drying, the electrode is covered with a dialysis membrane (Spectra/Por MWCO 6,000 - 8,000) fixed with an O-ring. The biosensor is immersed in 1mM phosphate buffer (pH 7.35 containing 0.1 M sodium chloride). It is then polarized at 200 mV (versus SCE). Then several aliquots of standard solutions of urea (5 mg/ml) are added to the measuring buffer. The current changes are recorded. The relationship between the urea concentration and the current change is illustrated in Figure 10.

This biosensor allows to perfom 30 reproducibile measurements.

This biosensor is tested after storage in dried state at temperature of 22±2°C under controlled umidity (<0.5%). After 6 month the sensitivity variation is not significant (<3%).

EXAMPLE 12

Preparation of the biosensor for the determination of oxalacetate by using a solid composite electrode modified with oxalacetate decarboxylase and dissolved hematein.

The solid composite electrode is described in Example n. 2. The oxalacetate decarboxylase (EC 4.1.1.3., ICN, Cat. No. 156007, 5,3 U) is applied onto the electrode surface. After drying, the electrode is covered with a dialysis membrane (Spectra/Por MWCO 6,000 - 8,000) fixed by means of an 0-ring. The biosensor is then plunged into 1 mM phosphate buffer (pH 8.0) containing 0.5 mM hematein and 0.1 M sodium chloride. It is then polarized at 0.0 mV (versus SCE). Several aliquots of standard solutions of sodium oxalacetate (20 mg/ml) are added to the measuring buffer. The current changes are recorded. The relationship between the oxalacetate concentration and the current change is shown in Figure 11.

EXAMPLE 13

Preparation of the biosensor based on a solid composite electrode modified with glucose oxidase and covered with a poly(para-phenylendiamine) film.

The solid composite electrode with the thick poly(para-phenylendiamine) film is prepared as described in Example N.5. The glucose oxidase (EC 1.1.3.4, Sigma, Cat. No. G-7016, 2 μl, 10 mg/ml) is applied onto the electrode surface that is then rinsed and covered with a dialysis membrane (Spectra/Por MWCO 6,000 - 8,000)

fixed with an O-ring. The biosensor is then immersed in a phosphate buffer (1mM, pH 7.0) containing 0.1 M sodium chloride. It si then polarized at -600 mV (versus SCE). Several aliquots of glucose standard solutions (20 mg/ml) are added to the measuring buffer. The current changes are recorded. The relationship between the glucose concentration and the current change is shown in Fig. 12.

EXAMPLE 14

Preparation of the biosensor for the determination of hydrogen carbonate based on a platinum electrode modified with carbonic anhydrase and dissolved hematein.

A solution of carbonic anhydrase (EC 4.2.1.1, Sigma, Cat. No. C 4831, 2400 W-A units, 2μl, 100 mg/ml) is applied onto the surface of the platinum electrode. After drying, the electrode is covered with a dialysis membrane (Spectra/Por MWCO 6,000 - 8,000) fixed by means of an O-ring. The biosensor is then immersed in Tris-HCl 4 mM (pH 8.30) containing 0.5 mM hematein and sodium chloride. It is then polarized at 0.0 mV (versus SCE). A few aliquots of sodium hydrogen-carbonate standard solutions (10 mg/ml) are added to the mesauring buffer. The relationship between the concentration of hydrogen carbonate and the current change is shown in Figure 13.

EXAMPLE 15

Preparation of the biosensor based on a platinum working electrode modified with penicillinase and dissolved hematein.

A solution (2 μl, 100 mg/ml) of penicillinase (EC 3.5.2.6, Sigma, Cat. No P-0389) is applied onto the platinum electrode surface. After having dried it at room temperature, the electrode is covered with a dialysis membrane (Spectra/Por MWCO 6,000 - 8,000) fixed by an O-ring. The biosensor is then immersed in 1 mM phosphate buffer (pH= 7.5) containing 0.5 mM hematein and 0.1 M sodium chloride. It is then polarized at 0.0 mV (versus SCE). A few aliquots of standard solutions of benzylpenicilline sodium salt (20 mg/ml) are added to the measuring solution. The current changes are recorded. The relationship between the benzylpenicilline and the current change is illustrated in Figure 14.

EXAMPLE 16

Preparation of the biosensor for ATP determination based on a platinum working electrode modified with apyrase and dissolved hematein.

RECTIFIED SHEET (RULE 91)

One solution (2μl, 200 mg/ml) of apyrase (EC 3.6.1.5, Sigma, Cat. No A- 6132) is applied onto the platinum electrode surface. After having dried it at room temperature, the electrode is covered with a dialysis membrane (Spectra/Por MWCO 6,000 - 8,000) fixed by an O-ring. The biosensor is immersed in Tris-HCl 2 mM (pH=7.0) containing 0.5 mM of hematein and 0.25 of sodium chloride. It is then polarized at 0.0 mV (versus SCE). Several aliquots of standard solutions of ATP sodium salt (20/ml) are added to the measuring buffer. The current changes are recorded. The relationship between the ATP concentration and the current change is illustrated in Figure 15.

EXAMPLE 17

Preparation of the biosensor for urea determination based on a golden electrode modified with methylene blue

The electrode is prepared as described in Example n. 3. Urease (3 μl, 10 mg/ml) is applied onto the electrode surface. After drying, the electrode is covered with a dialysis membrane (Spectra/Por MWCO 6,000 - 8,000) fixed by an O-ring. The biosensor is immersed in 1 mM phosphate buffer (pH = 7.50) containing 0.1 M sodium chloride. It is then polarized at -100 mV (versus SCE). A few aliquots of urea standard solution (5mg/ml) are added to the measuring buffer. The current changes are recorded. The relationship between the urea concentration and the current change is shown in Figure 16.

CLAIMS

1. An amperometric biosensor system for the detection of analytes comprising:
a) at least one biocatalyst producing a pH change by its interaction with the analyte;
b) at least one compound exhibiting different redox properties in its protonated and non-protonated forms (pH-sensitive redox compounds) selected in the group consisting of cyclic hydrocarbons, containing from 4 to 30 carbon atoms and susbstituted with at least one group selected from -OH, -SH, - NH_2, =O, =S, =NH, - OR_1, - SR_1, - NHR_1, - NR_1R_2, = NR_1, wherein R_1 and R_2 are hydrocarbon chains optionally further substituted, or selected in the group consisting of heterocyclic compounds containing from 3 to 30 carbon atoms and one or more heteroatoms selected in the group consisting of N, S, O, Se, Te, B, P, As, Sb, Si, optionally substituted with a group selected from -OH, -SH, - NH_2, =O, =S, =NH, -OR, - SR_1, - NHR_1, - NR_1R_2, = NR_1, wherein R_1 and R_2 are independent hydrocarbon chains;
c) a working electrode;
d) a reference electrode;
being said electrodes connected through an ammeter.

2. The biosensor system according to claim 1, wherein said biocatalyst is selected in the group consisting of enzymes, synzymes, cells, cell components, tissues, imunoproteins, nucleic acids and extracts, fractions, fragments, homogenates, lysates thereof.

3. The biosensor system according to claim 2, wherein said enzyme is selected in the group consisting of hydrolase, oxydoreductase, transferase, lyase, ligase.

4. The biosensor system according to claim 2, wherein said enzyme is selected in the group consisting of phospshorylase, decarboxylase, esterase, phosphatase, deaminase.

5. The biosensor system according to claim 2, wherein said enzyme is selected in the group consisting of urease, oxalacetate decarboxylase, glucose oxidase, carbonic anhydrase, penicillinase, apyrase.

6. The biosensor system according to claim 1-5, wherein said pH-sensitive redox compound is in the form of a monomer, oligomer or polymer.

7. The biosensor system according to claims 1-6 wherein said pH-sensitive redox

compound (b) is selected among the pH indicators, phenoxazines and phenothiazines dyes, and natural antioxidants.

8. The biosensor system according to claim 7, wherein said pH-sensitive redox compound (b) is selected in the group consisting of hematoxylin, hematein, methylene blue, quercitin, flavonoids, alkyl gallates, polymerized ortho-phenylenediamine or para-phenylendiamine.

9. The biosensor system according to claims 1-8, wherein said working electrode (c) is a solid composite electrode, or platinum electrode , or gold electrode, or mercury electrode or glassy carbon electrode.

10. The biosensor system according to claims 1-9 wherein said reference electrode (d) is selected in the group consisting of Ag/AgCl and calomel electrodes.

11. A method for the determination of analytes characterized by the use of a biosensor as claimed in claims 1-10.

12. A method according to claim 11, wherein said method consists in:

(a) placing the electrodes in a measuring solution;

(b) applying a suitable potential between the electrodes;

(c) measuring a background current;

(d) adding to the solution the sample containing the analyte to be determined;

(e) measuring the current change that is proportional to the analyte concentration;

(f) optionally subtracting the current change measured with a blank electrode from the value obtained in (e).

13. A method according to claim 11, wherein said method consists in:

(a) applying a suitable potential between the electrodes;

(b) measuring a background current;

(c) contacting the biosensor with the sample containing the analyte;

(d) measuring a current change that is proportional to the analyte concentration;

(e) optionally subtracting the current change measured with a blank electrode from the value obtained in (d)

14. A method according to claim 11, wherein said biocatalyst contained in the biosensor system is selected among the biocatalysts that are inhibited by said analyte, said method consisting in:

(a) placing the electrodes in a measuring solution;
(b) applying a suitable potential between the electrodes;
(c) adding the substrate of said biocatalyst to the measuring solution;
(d) measuring a background current;
(e) adding to the solution the sample containing the inhibiting-analyte to be determined;
(f) measuring a current change that is proportional the inhibiting-analyte concentration;
(g) optionally subtracting the current change measured with a blank electrode from the value obtained in (f).

15. A method according to claim 11, wherein said biocatalyst contained in the biosensor system is selected among the biocatalysts that are inhibited by said analyte said method consisting in:
(a) applying a suitable potential between the electrodes;
(b) adding the substrate of said biocatalyst;
(c) measuring a background current;
(d) contacting the biosensor with the sample containing the inhibiting-analyte system;
(e) measuring a current change that is proportional to the inhibiting-analyte concentration;
(f) optionally subtracting the current change measured with a blank electrode from the value obtained in (e).

16. Use of the biosensor system as claimed in claims 1-10 for the amperometric detection of analytes in human and veterinary diagnostics, industrial processes, agro-food industry, pharmaceutical industry, environmental monitoring.

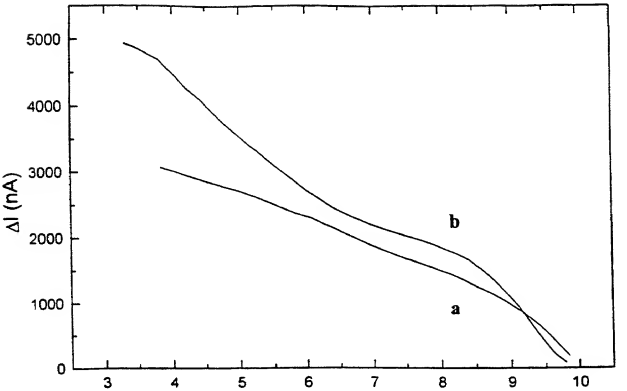
5000
4000
3000
2000
1000
0
ΔI (nA)
3
4
5
6
7
8
9
10
pH
a
b

Fig. 1

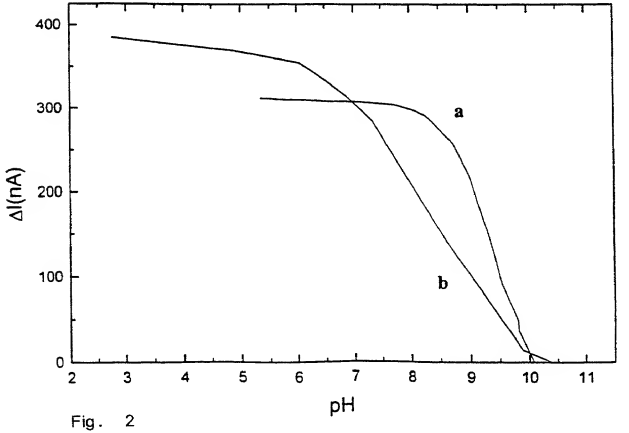
400
300
200
100
0
ΔI(nA)
2
3
4
5
6
7
8
9
10
11
pH
a
b

Fig. 2

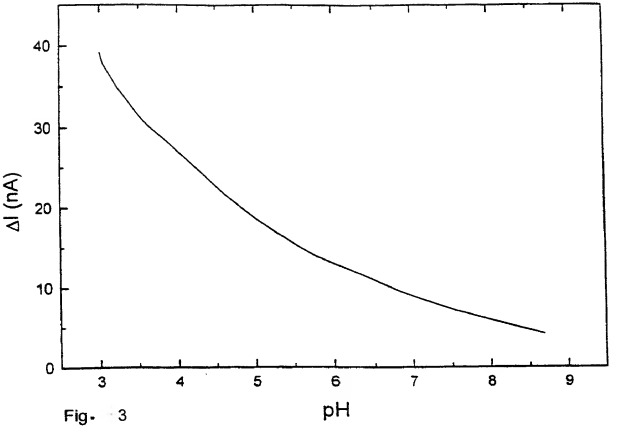
ΔI (nA)
pH
0
10
20
30
40
3
4
5
6
7
8
9

Fig. 3

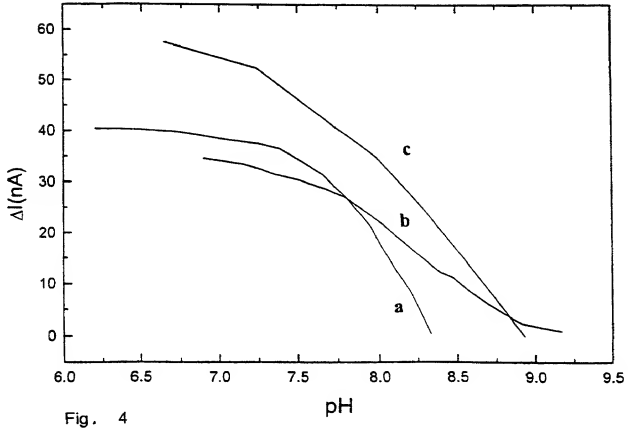
ΔI(nA)
pH
a
b
c
0
10
20
30
40
50
60
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5

Fig. 4

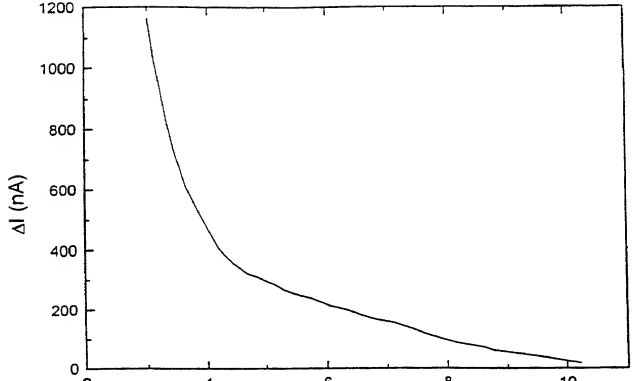
1200
1000
800
600
400
200
0
ΔI (nA)
2
4
6
8
10
pH

Fig. 5

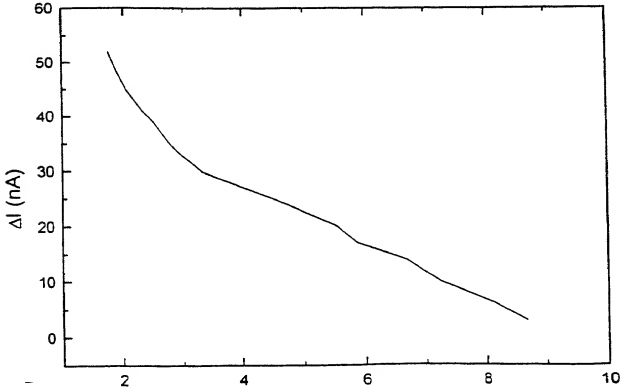
60
50
40
30
20
10
0
ΔI (nA)
2
4
6
8
10
pH

Fig. 6

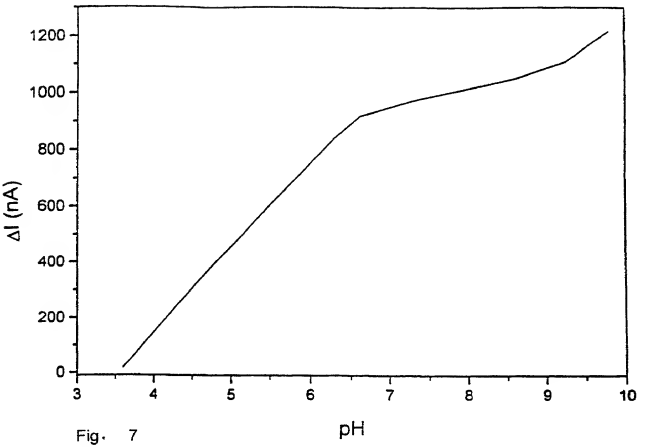
1200
1000
800
600
400
200
0
ΔI (nA)
3
4
5
6
7
8
9
10
pH

Fig. 7

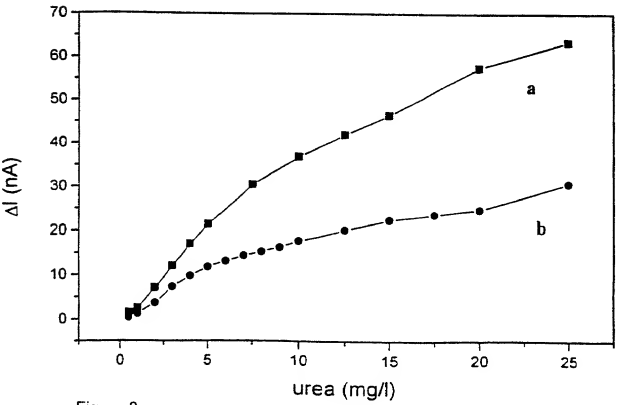
70
60
50
40
30
20
10
0
ΔI (nA)
a
b
0
5
10
15
20
25
urea (mg/l)

Fig. 8

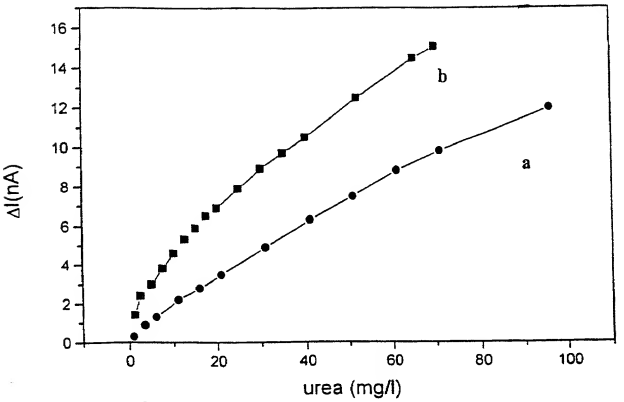

Fig. 9

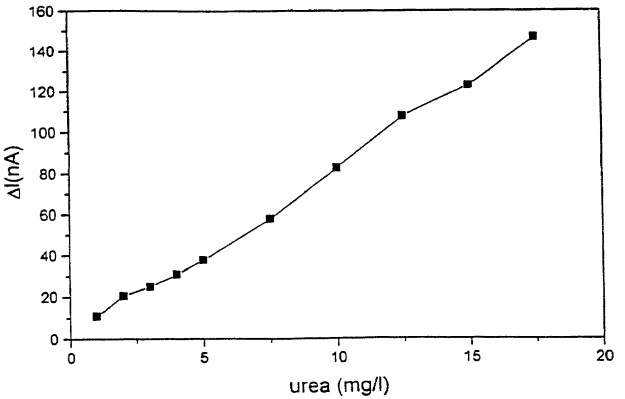

Fig. 10

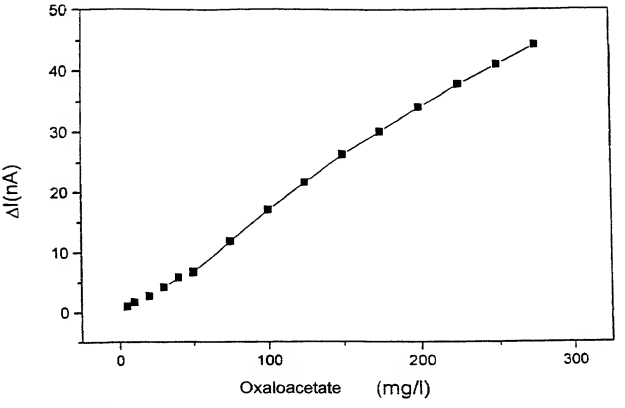
50
40
30
20
10
0
ΔI(nA)
0
100
200
300
Oxaloacetate (mg/l)

Fig. 11

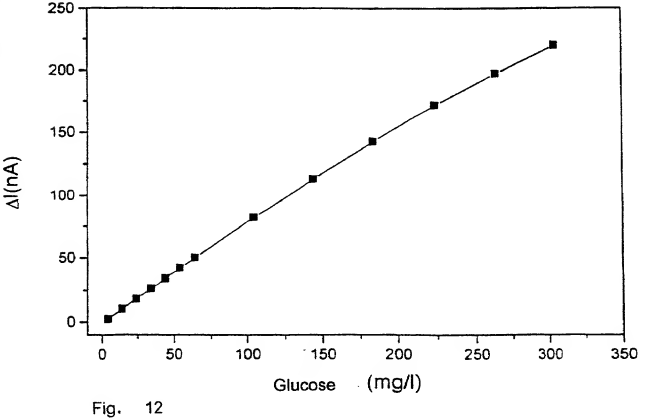
250
200
150
100
50
0
ΔI(nA)
0
50
100
150
200
250
300
350
Glucose (mg/l)

Fig. 12

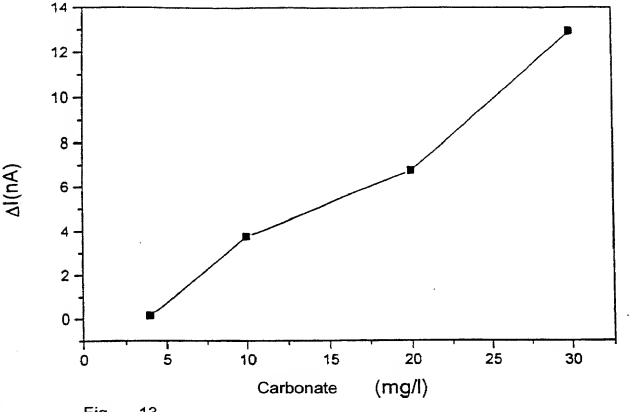
ΔI(nA)
0
2
4
6
8
10
12
14
0
5
10
15
20
25
30
Carbonate (mg/l)

Fig. 13

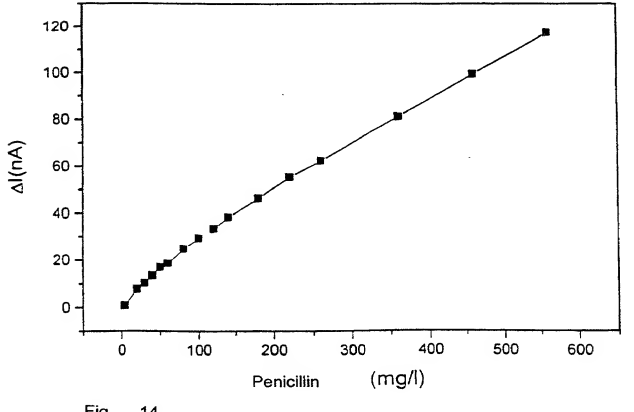
ΔI(nA)
0
20
40
60
80
100
120
0
100
200
300
400
500
600
Penicillin (mg/l)

Fig. 14

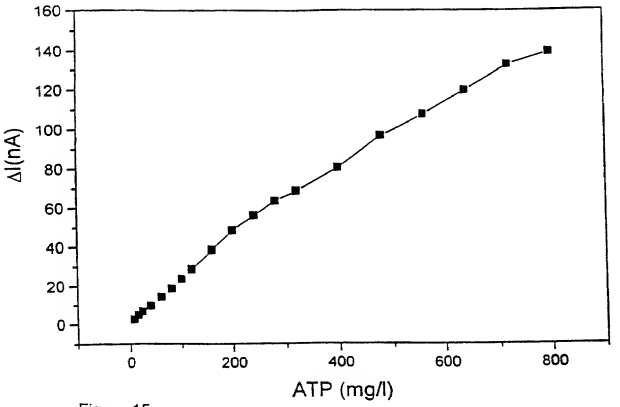
160
140
120
100
80
60
40
20
0
ΔI(nA)
0
200
400
600
800
ATP (mg/l)

Fig. 15

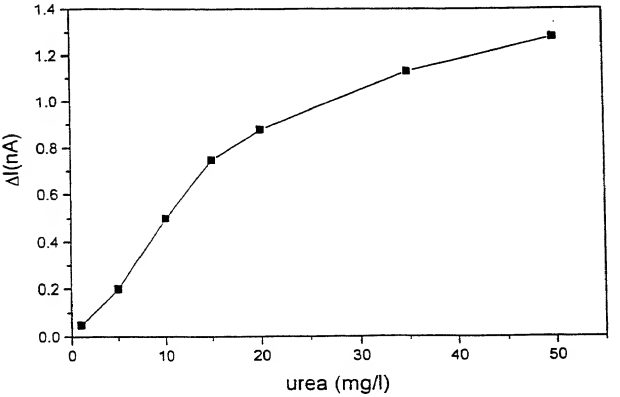
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ΔI(nA)
0
10
20
30
40
50
urea (mg/l)

Fig. 16

Docket No.

COMBINED DECLARATION AND POWER OF ATTORNEY FOR ORIGINAL, DESIGN, NATIONAL STAGE OF PCT, SUPPLEMENTAL, DIVISIONAL, CONTINUATION OR CONTINUATION-IN-PART APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

PH-SENSITIVE AMPEROMETRIC BIOSENSOR

the specification of which

a. ☐ is attached hereto

b. ☐ was filed on as application Serial No. and was amended on . (if applicable).

PCT FILED APPLICATION ENTERING NATIONAL STAGE

c. ☒ was described and claimed in International Application No. PCT/EP00/00455 filed on 21 January 2000 and as amended on 20 December 2000 . (if any).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. § 1.56.

I hereby specify the following as the correspondence address to which all communications about this application are to be directed:

SEND CORRESPONDENCE TO:

MORGAN & FINNEGAN, L.L.P.
345 Park Avenue
New York, N.Y. 10154

DIRECT TELEPHONE CALLS TO:

☒ I hereby claim foreign priority benefits under Title 35, United States Code § 119 (a)-(d) or under § 365(b) of any foreign application(s) for patent or inventor's certificate or under § 365(a) of any PCT international application(s) designating at least one country other than the U.S. listed below and also have identified below such foreign application(s) for patent or inventor's certificate or such PCT international application(s) filed by me on the same subject matter having a filing date within twelve (12) months before that of the application on which priority is claimed:

☒ The attached 35 U.S.C. § 119 claim for priority for the application(s) listed below forms a part of this declaration.

Country/PCT	Application Number	Date of filing (day, month, yr)	Date of issue (day, month, yr)	Priority Claimed
ITALY	MI99A000210	4 February 1999		☒ Y ☐ N
				☐ Y ☐ N
				☐ Y ☐ N

☐ I hereby claim the benefit under 35 U.S.C. § 119(e) of any U.S. provisional application(s) listed below.

Provisional Application No.	Date of filing (day, month, yr)

ADDITIONAL STATEMENTS FOR DIVISIONAL, CONTINUATION OR CONTINUATION-IN-PART OR PCT INTERNATIONAL APPLICATION(S DESIGNATING THE U.S.)

I hereby claim the benefit under Title 35, United States Code § 120 of any United States application(s) or under § 365(c) of any PCT international application(s) designating the U.S. listed below.

US/PCT Application Serial No.	Filing Date	Status (patented, pending, abandoned)/ U.S. application no. assigned (For PCT)

US/PCT Application Serial No.	Filing Date	Status (patented, pending, abandoned)/ U.S. application no. assigned (For PCT)

☐ In this continuation-in-part application, insofar as the subject matter of any of the claims of this application is not disclosed in the above listed prior United States or PCT international application(s) in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

I hereby appoint the following attorneys and/or agents with full power of substitution and revocation, to prosecute this application, to receive the patent, and to transact all business in the Patent and Trademark Office connected therewith: John C. Vassil (Reg. No. 19,098), Alfred P. Ewert (Reg. No. 19,887), David H. Pfeffer (Reg. No. 19,825), Harry C. Marcus (Reg. No. 22,390), Robert E. Paulson (Reg. No. 21,046), Stephen R. Smith (Reg. No. 22,615), Kurt E. Richter (Reg. No. 24,052), J. Robert Dailey (Reg. No. 27,434), Eugene Moroz (Reg. No. 25,237), John F. Sweeney (Reg. No. 27,471), Arnold I. Rady (Reg. No. 26,601), Christopher A. Hughes (Reg. No. 26,914), William S. Feiler (Reg. No. 26,728), Joseph A. Calvaruso (Reg. No. 28,287), James W. Gould (Reg. No. 28,859), Richard C. Komson (Reg. No. 27,913), Israel Blum (Reg. No. 26,710), Bartholomew Verdirame (Reg. No. 28,483), Maria C.H. Lin (Reg. No. 29,323), Joseph A. DeGirolamo (Reg. No. 28,595), Michael P. Dougherty (Reg. No. 32,730), Seth J. Atlas (Reg. No. 32,454), Andrew M. Riddles (Reg. No. 31,657), Bruce D. DeRenzi (Reg. No. 33,676), Mark J. Abate (Reg. No. 32,527), John T. Gallagher (Reg. No. 35,516), Steven F. Meyer (Reg. No. 35,613) and Kenneth H. Sonnenfeld (Reg. No. 33,285), Tony V. Pezzano (Reg. No. 38,271), Andrea L. Wayda (Reg. 43,979), Walter G. Hanchuk (Reg. No. 35,179), John W. Osborne (Reg. No. 36,231), and Robert K. Goethals (Reg. No. 36,813) of Morgan & Finnegan, L.L.P. whose address is: 345 Park Avenue, New York, New York, 10154; and Michael S. Marcus (Reg. No. 31,727), John E. Hoel (Reg. No. 26,279), and Stanley B. Green (Reg. No. 24,351) of Morgan & Finnegan, L.L.P., whose address is 1775 Eye Street, Suite 400, Washington, D.C. 20006.

☐ I hereby authorize the U.S. attorneys and/or agents named hereinabove to accept and follow instructions from as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. attorneys and/or agents and me. In the event of a change in the person(s) from whom instructions may be taken I will so notify the U.S. attorneys and/or agents named hereinabove.

Full name of sole or first inventor:

Inventor's signature* _____ 20 July 2001

Andrea PIZZARIELLO Date

Residence: Via Friuli 14 - 31057 SILEA - ITALY

Citizenship: ITALIAN

Post Office Address: Via Friuli 14 - 31057 SILEA - ITALY

Full name of second inventor:

Inventor's signature* _____ 20 July 2001

Miroslav STREDANSKY Date

Residence: Komenskeho 13 - 900 01 MODRA - SLOVAK REPUBLIC

Citizenship: SLOVAK

Post Office Address: Komenskeho 13 - 900 01 MODRA - SLOVAK REPUBLIC

☒ ATTACHED IS ADDED PAGE TO COMBINED DECLARATION AND POWER OF ATTORNEY FOR SIGNATURE BY THIRD AND SUBSEQUENT INVENTORS FORM.

Full name of third inventor:

Inventor's signature* S Stredanska — 20 July 2001

Silvia STREDANSKA — Date

Residence: Komenskeho 13 - 900 01 MODRA - SLOVAK REPUBLIC

Citizenship: SLOVAK

Post Office Address: Komenskeho 13 - 900 01 MODRA - SLOVAK REPUBLIC

Full name of fourth inventor:

Inventor's signature* S. Miertus — 20 July 2001

Stanislav MIERTUS — Date

Residence: Pod Rovnicami 27 - 841 05 BRATISLAVA - SLOVAK REPUBLIC

Citizenship: SLOVAK

Post Office Address: Pod Rovnicami 27 - 841 05 BRATISLAVA - SLOVAK REPUBLIC

Full name of fifth inventor:

Inventor's signature* _____ _____

Date

Residence:

Citizenship:

Post Office Address:

Full name of sixth inventor:

Inventor's signature* _____ _____

Date

Residence:

Citizenship:

Post Office Address:

Full name of seventh inventor:

Inventor's signature* _____ _____

Date

Residence:

Citizenship:

Post Office Address: